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IMMUNIZATION AGAINST PLAGUE BY A SPECIFIC FRACTION OF PASTEURELLA PSEUDOTUBERCULOSIS

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ABSTRACT

An antigen, designated PF, common to <u>Pasteurella pestis</u> and <u>Pasteurella pseudotuberculosis</u>, has been isolated free of antigens detectable by gel diffusion. It will protect guinea pigs against plague one day after vaccination. PF apparently is a protein-lipopolysaccharide complex and is analogous to endotoxin in increasing resistance to infection, but is nontoxic in mice and guinea pigs. PF is different from and does not contain any of the antigens that have been shown previously to protect against plague.

ACKNOWLEDGMENT

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I. INTRODUCTION

It has long been known that vaccination with Pasteurella pseudotuberculosis can immunize guinea pigs against challenge with virulent Pasteurella pestis.[1] Several investigators[2-5] have observed antigens common to both organisms, but a specific antigen responsible for protection against plague has not been determined. Our investigation resulted in the identification and partial purification of an antigen, designated PF, from an avirulent P. pseudotuberculosis strain that can protect guinea pigs against plague as early as one day after vaccination.*

II. METHODS

Initial attempts to purify the protective factor (PF) from avirulent P. pseudotuberculosis strain 4-C (Type I) demonstrated that, after sonication and centrifugation, all of the protective material was associated with the residue. The next step was assisted by a publication by Keppie, Cooking, and Smith,[6] which stated that a nontoxic complex from P. pestis that immunized mice and guinea pigs could be isolated by ultrasonic oscillation followed by solubilization of the residue in 0.05 M NaHCO$_3$ buffer, pH 8.5. Application of this treatment to P. pseudotuberculosis caused all of the protective activity to go into "solution," which suggested that our protective factor and Keppie's immunizing complex may contain the same antigen.

We tried several purification procedures, including continuous flow curtain electrophoresis and chromatography on DEAE cellulose, but the results showed no significant purification. The most successful procedure was centrifugation at 100,000 x g in the Spinco Model L ultracentrifuge. This resulted in a dark precipitate that contained all of the protective factor. Since most of the soluble antigens remained in the supernate, a few washes in bicarbonate buffer followed each time with ultracentrifugation resulted in an opalescent product free of antigens detectable by gel diffusion. This product is referred to as purified PF. The final method used to obtain purified PF was:

(a) Bacteria were grown at 26°C for one day in a two-liter flask containing 300 milliliters of Difco heart infusion broth supplemented with 0.5 per cent xylose.

(b) Cells were sedimented by centrifugation at 2000 x g for ten minutes.

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* In conducting the research reported herein, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

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(c) Cells were resuspended in a 1:20 volume of distilled water and sonicated in a ten-kilocycle Raytheon Sonic Oscillator for 30 minutes at maximum plate voltage. Sonicated material was centrifuged at 39,000 x g for ten minutes.

(d) The precipitate was washed in approximately ten volumes of distilled water and resedimented by centrifugation at 39,000 x g for ten minutes.

(e) The precipitate was resuspended in 0.05 M sodium bicarbonate buffer, pH 8.5, and resonicated for 30 minutes at maximum plate voltage. Sonicated material was centrifuged at 2000 x g for ten minutes.

(f) The supernatant fluid was centrifuged at 44,000 x g for 60 minutes in a Spinco Model L ultracentrifuge.

(g) The precipitate was resuspended in approximately 100 volumes of 0.05 M sodium bicarbonate buffer, pH 8.5, and recentrifuged at 44,000 x g for 60 minutes. This step was repeated three times to wash the sedimentable PF free from nonsedimenting antigens.

(h) The washed suspension of PF was sterilized by heating at 60°C for 30 minutes and lyophilized.

The procedure for assaying purified PF was as follows: A sample was mixed 50:50 with incomplete Freund's adjuvant, and one milliliter was injected intramuscularly into the hind leg of a guinea pig. Animals were challenged via the peritoneal cavity with approximately 5000 organisms of the virulent Alexander strain of P. pestis (500 times the LD_{50} dose) and observed for 21 days.

Purified PF was assayed for protein by the method of Lowry,[7] for carbohydrate by the method of Shields and Burnett,[8] and for lipid by the method of Snyder and Stephens.[9]

III. RESULTS

Initially, PF was assayed by challenging guinea pigs three weeks after vaccination. In order to determine how soon immunity developed after vaccination, purified PF was prepared from several strains of P. pseudotuberculosis and P. pestis, and immunity was tested at one, two, and three weeks after vaccination. The results of this experiment (Table I) indicated that PF was as protective one week after vaccination as it was three weeks after vaccination. Although not shown in Table I, PF from P. pseudotuberculosis Type IV gave complete protection five weeks after vaccination. This strain was used to determine whether immunity was developed in less than one week.

TABLE I. EFFECTIVENESS OF DIFFERENT PF PREPARATIONS IN PROTECTING GUINEA PIGS AGAINST PLAGUE

PF Source	Time of Challenge[a]/ Post-Vaccination					
	1 week		2 weeks		3 weeks	
	10 mg PF	1 mg PF	10 mg PF	1 mg PF	10 mg PF	1 mg PF
P. pseudotuberculosis strains:						
4C	1/4(13)[b]	3/3(12)	2/4(12)	4/4(10)	4/4(11)	4/4(10)
I(2)	3/4(15)	3/4(12)	3/4(12)	4/4(10)	2/4(14)	4/4(9)
II(16)	2/4(11)	4/4(11)	1/4(12)	4/4(12)	2/4(13)	3/4(11)
III(43)	1/4(13)	3/4(12)	0/4	4/4(12)	1/4(21)	3/4(10)
IV	0/4	0/4	Not Tested		0/4	0/4
V(25)	4/4(11)	4/4(9)	3/4(13)	4/4(9)	Not Tested	
P. pestis strains:						
TRU	2/4(11)	4/4(10)	1/4(12)	3/4(11)	2/4(13)	4/4(10)
DI	4/4(13)	4/4(11)	2/4(14)	4/4(13)	Not Tested	
EV76	2/4(16)	3/4(13)	2/4(13)	3/4(12)	0/4	3/4(11)
M23	Not Tested	Not Tested	0/4	2/4(15)	0/4	2/4(14)
14	Not Tested	Not Tested	3/4(14)	3/4(11)	1/4(8)	1/4(13)
Controls	18/20(7)		20/20(8)		20/20(8)	

a. Challenge dose ranged from 2300 to 6500 P. pestis strain Alexander injected intraperitoneally.

b. Results are expressed as Dead/Total (average day of death). The average day of death was calculated as the sum of the days after challenge that each death occurred divided by the total number of dead animals.

The results of challenging guinea pigs one to seven days after vaccination (Table II) showed that immunity was present as soon as one day. Although the number of guinea pigs in each group was small, protection one day after vaccination has been observed with each of five similar, but not identical, batches of PF.

TABLE II.   EFFECTIVENESS OF PURIFIED PF FROM P. PSEUDOTUBERCULOSIS IV
IN PROTECTING GUINEA PIGS AGAINST PLAGUE
ONE TO SEVEN DAYS POST-VACCINATION

| Day of Challenge[a]/ | Dead/Total (average day of death) | |
Post-Vaccination	Controls	Vaccinated[b]/
1	4/4(9)	0/4
2	4/4(9)	1/4(6)
3	4/4(9)	1/4(10)
4	4/4(10)	0/4
5	4/4(9)	0/4
6	Not Tested	0/4
7	4/4(7)	0/4

a. Challenge dose = 5000 P. pestis strain Alexander injected intra-
peritoneally.
b. All animals were vaccinated intramuscularly with 10 milligrams
of PF prepared from P. pseudotuberculosis IV mixed with incomplete
Freund's adjuvant. Controls received 0.05 M sodium bicarbonate
buffer mixed with adjuvant.

Chemical assay of purified PF accounted for 96 per cent of the non-dialyzable dry weight as follows: 65 per cent protein, 12 per cent lipid, and 19 per cent carbohydrate. Recent data obtained by density gradient centrifugation suggest that "purified PF" may contain more than one component, which brings into question the significance of relating the chemical assays to the protective factor.

When purified PF was diffused in a gel plate against several complex P. pestis and P. pseudotuberculosis antisera, no bands of precipitate were evident. Furthermore, injection of PF into a rabbit yielded antiserum that showed no bands against PF but one strong band against sonicated cell supernate. If anti-PF serum was adsorbed with PF, this band was removed. The present hypothesis to explain these observations is that (a) PF antigen is too large to diffuse in a gel plate; and (b) sonic oscillation splits a

portion of the large PF molecule into a fragment that can diffuse into agar and react with anti-PF serum. Additional evidence for this hypothesis is that, after treatment with sodium deoxycholate, purified PF will react in a gel plate with anti-PF serum. Sodium deoxycholate treatment also results in the liberation into the supernate of about 50 per cent of the total protein in purified PF and in the destruction of the protective activity.

By diffusing anti-PF against several of the standard antigen antibody systems described in the past,[6] the antibody coprecipitating PF was shown to be the same as the antibody directed against the lipopolysaccharide identified and purified by Davies.[10] He demonstrated that the purified lipopolysaccharide was not protective in animals.

Attempts to correlate immunity with a specific antibody obtained from immunized animals have not been successful. Anti-PF serum will not protect passively when injected into normal animals, and several immunized guinea pigs have had no antibodies detectable by gel diffusion methods.

IV. DISCUSSION

The finding that purified PF can elicit immunity against plague one day after vaccination brings to mind the analogous finding that injection of endotoxins from Gram-negative bacteria can increase resistance of animals within several hours to a variety of pathogens.[11-13] Certain differences are apparent. The toxic LD_{50} in mice of classical endotoxin is near one microgram; the toxic intraperitoneal LD_{50} of purified PF is more than five milligrams in mice and more than 20 milligrams in guinea pigs. Davies,[14] on the basis of carbohydrate analysis of somatic antigens, concluded that P. pestis did not contain a typical endotoxin but that P. pseudotuberculosis did. PF appears to be present in both species. Also, the resistance-enhancing property of endotoxin is evident after several hours and may last several days. The resistance-enhancing property of PF was still evident five weeks after injection. The latter observation is analogous to the data of Dubos and Schaedler,[15] which demonstrated that a resistance to mycobacterial and staphylococcal infections persisted for several weeks after administration of endotoxin. Endotoxin generally increases resistance to infections of bacterial species that are sensitive to the bactericidal action of serum, although there are some cases where increased resistance has been induced to bacterial species that were insensitive to the bactericidal action of serum.[16] P. pestis is not sensitive to bactericidal action in guinea pig serum according to Jawetz and Meyer[17] and to Janssen.*

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* Unpublished data.

The importance of PF is twofold. First, its effect should be considered in formulating any plague vaccine. Since the avirulent P. pseudotuberculosis strain used to obtain PF cannot produce any of the antigens presently implicated in plague immunity (i.e., Fraction I, V, W, and plague toxin), the resistance described in this paper must be due to an antigen different from Fraction I, V, W, or plague toxin. Second, although several differences between PF and endotoxin have been mentioned, the similarity of PF to endotoxin warrants further study to determine the specificity and mechanism of action of the increased resistance elicited by the injection of PF.

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